(54) Title: A METHOD FOR ANALYZING AN ORGANIC SAMPLE

(57) Abstract

Described is a method and apparatus for analyzing an organic sample. In the preferred embodiment, this method and apparatus allows the determination of the base sequence of a nucleic acid by determining the molecular weights of the components of a biological sample. The method uses either a pre-existing chromophore or the covalent attachment of an ionizable chromophore to a biological sample followed by the vaporization of these molecules by exposure to an intense pulse of electromagnetic radiation (14) in the presence of a matrix which strongly absorbs the radiation. The gaseous molecules are subsequently extracted into an evacuated ionization chamber (15) and then exposed to electromagnetic radiation (32) at a wavelength which specifically excites the chromophore covalently attached to the biological sample.

A METHOD FOR ANALYZING AN ORGANIC SAMPLE

Technical Field

5   The present invention relates, in general, to a method capable of identifying or determining the molecular weights of organic samples and, in particular, relates to the use of this method to construct an automated apparatus that can determine the sequence of nucleic acids utilizing non-isotopic and non-electrophoretic techniques.

10 ## Background Art

An important property of biological samples which often must be determined is their molecular weight. The most common method used to perform this measurement is to electrophorese the biomolecule through
15 an acrylamide or agarose gel, visualize the position in the gel by staining or autoradiography, and determine the sizes by comparison to molecular weight standards of known sizes.

A related technology which uses similar sizing
20 and detection techniques is DNA or RNA sequencing. DNA is a long thread-like macromolecule comprised of a chain of four deoxyribonucleotides which contain one of the four nitrogenous bases adenine (A), cytidine (C), guanine (G), or thymine (T). Similarly, RNA is composed
25 of a long chain of ribonucleotides. The order of these nucleotides is the genetic code of the organism from which the DNA was isolated. The determination of this order is, therefore, a most important goal for scientists working in biological fields.
30 Manual methods to sequence DNA involve either synthesis of new DNA in the presence of dideoxyribo-

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nucleotide terminators using as a template the DNA whose
sequence needs to be determined or the degradation of
the DNA to be sequenced using base-specific chemical
treatments. In each case, a nested set of radioactively
5   labelled DNA fragments are generated which represent the
sequence of the DNA. For example, in the dideoxy method
(Sanger, F. et al., (1977) PROC. NATL. ACAD. SCI. U.S.A., 74,
5463-5467), the template DNA, whose sequence is to be
determined, is incubated with an oligonucleotide primer,
10   four deoxyribonucleoside 5'-triphosphates (dATP, dCTP,
dGTP and dTTP), and a DNA polymerase. The primer
anneals to a specific complementary position in the
template DNA that is defined by the order of the bases
in the primer. The DNA polymerase then begins to
15   catalyze DNA synthesis in the 5' to 3' direction by
incorporating the deoxyribonucleoside 5'-triphosphate
that is complementary to the next base in the template
DNA. A complementary nucleotide is defined as one that
follows the base-paring rules which require an A of one
20   strand of a double-stranded DNA molecule always pairs
with a T of the other strand and that a C of one strand
always pairs with a G of the other strand.

In addition to the ability of DNA polymerases
to incorporate normal nucleotides into the newly synthe-
25   sized strand, many polymerases can also incorporate
dideoxyribonucleoside 5'-triphosphates. Dideoxyribon-
ucleotides are identical to deoxyribonucleotides except
that they lack the 3'-hydroxy group on the ribose sugar.
When these nucleotide analogs are incorporated into a
30   growing DNA chain, synthesis terminates because the
chain no longer bears the 3'-hydroxyl needed to add
subsequent nucleotides. In the dideoxy sequencing
method, four separate sequencing reactions are run, each
containing one of the four dideoxyribonucleotides (each
35   reaction also contains the four normal deoxyribonu-
cleotides, one labeled with ^{32}P or ^{35}S). Incorporation of

the dideoxy analogs occurs occasionally and randomly in place of a normal nucleotide at complementary positions in the template so that each reaction generates a heterogeneous population of product DNA molecules each
5 beginning with the primer (and thus sharing a common 5'-terminus) and each terminating with the dideoxynucleotide that was included in that reaction.

The radioactively labeled products from each of the four dideoxy sequencing reactions are denatured
10 to separate the newly synthesized DNA from the template and then electrophoresed in adjacent lanes on a poly-acrylamide gel such that the DNA product molecules are separated based on their chain length. The presence of a band in the gel represents the presence of the corre-
15 sponding complementary nucleotide in the template at a specific distance from the primer. Comparison and analysis of the bands present in each of the four lanes allows the sequence of the template DNA to be deduced. Thus, the relative positions of the bands identify the
20 positions in the DNA sequence of each given nucleotide base. Generally, the DNA molecules are labelled so that the bands produced are readily detected. As shown in Figure 1, the intensity of the bands is generally non-uniform, within a single lane, because band intensity is
25 directly related to the total number or concentration of DNA molecules of the same molecular weight in a specific lane, and this number varies from one molecule to another even when they are of approximately the same molecular weight and even when they contain the same
30 chain terminating agent. Tabor and Richardson, U.S. Patent 4,962,020, have recently described a method for producing uniform banding, by use of a manganese buffer.

In the alternative chemical DNA sequencing method, chemicals that effect random partial cleavage of
35 the DNA at G, G+A, C+T, and C are added in four individual reactions to a single-stranded DNA fragment contain-

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ing a ^{32}P label at the 5' end.   The resulting fragments
are processed as in the dideoxy method to determine the
DNA sequence.   Maxam, A. and Gilbert, W. (1977) PROC.
NATL. ACAD. SCI. U.S.A., 74, 560-564.

5   Automated DNA sequencing instruments based on
the dideoxy method are described in U.S. Patent Nos.
4,855,255 and 4,811,218 and Prober et al., Science   16
Oct. 1987, 238; pp. 336-341.   Both of these systems
require the incorporation of four fluorescent dyes into
10   the dideoxy-terminated product DNA which are then run on
a polyacrylamide gel.   The discrete-length product
molecules are detected near the bottom of the gel by
their emitted florescence following excitation with a
laser.   In these automated systems, many more sequences
15   can be analyzed per gel and the sequences determined
accurately out to 500 bases or greater.   Furthermore,
data can be recorded faster since there is no manual gel
reading step required.   Finally, the automated sequen-
cers use non-isotopic detection methods so there is not
20   added costs associated with radioactive wasted disposal.

Fluorescent labels can be used in place of
radioactive labels, as described in Fung et al., U.S.
Patent 4,855,225 and Hunkapiller et al. 4,811,218, and
Prober et al., 238 Science 336, 1987.   In addition, the
25   DNA molecules may be labeled with different isotopic
variants of an atom, e.g., sulfur.   The sulfur atom is
used as a marker for the specific nucleotide at the end
of each nucleic acid molecule, and later identified by
combustion of the molecule to produce sulfur dioxide,
30   which is then detected using mass spectrometry.
Brennan, U.S. Patent No. 5,003,059; Jacobson et al.,
U.S. Patent 5,002,868; and Serge, EPA 0 360 676 A1.

Although these instruments offer some advan-
tages over manual methods, they still suffer from
35   numerous drawbacks which are inherent in the use of a
polyacrylamide gel to resolve the DNA fragments.   For

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example, this method remains labor-intensive since a gel must be poured and disposed of for each sequencing run. Also, the accuracy of the sequencing can be impacted by artifacts generated by non-uniform gel matrix or even by
5   a particular sequence as it electrophoreses down the gel.   Furthermore, although more sequences can be determined on one gel that can be done manually, 10 to 12 hours are still required to obtain this data.

These problems associated with sequencing are
10 minor when one is considering the generation of the sequence of a small genome, but they become monumental when contemplating sequencing the human genome, estimated to contain over 3 billion base pairs.

Mass spectral methods are well known.   Pulsed
15 mass spectroscopic methods, Burlingame, A.L. et al., (1990) ANAL. CHEM., 62, 268R-303R (and references therein), such as time-of-flight (TOF) and Fourier transform ion-cyclotron-resonance mass spectroscopy (FTICR-MS), have the inherent ability to simultaneously analyze all
20 of the components of a complex mixture in a single 200 millisecond experiment. The most significant feature of a mass spectroscopic-based method is that it does not require prior electrophoretic or chromatographic separation prior to analysis thus reducing the analysis time
25 by at least three orders of magnitude.

A major obstacle, until now, for implementing mass spectroscopy for analysis of large biomolecules has been the lack of an appropriate interface between the water-based biological system and the high vacuum
30 required for mass analysis.   Prior studies have used techniques such as secondary ion mass spectroscopy, Aberth, W. et al., (1982) ANAL. CHEM., 54, 2029-2034, fast atom bombardment, Griffen, D. et al., (1989) BIOMED. & ENV. MASS SPECTROM., 17, 105, [252] Cf plasma desorption,
35 Sundqvist, B. et al., (1985) MASS SPECTROM, REV., 4, 421-460, electrospray, Fenn, J.B. et al., (1990) MASS SPECTROM

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REVIEWS, 9, 37-70, and thermospray, Straub, K. et al.,
(1990) RAPID COMMUN. MASS SPECTROM, 4, 267-271, Pramanik,
B.C. et al., (1989) ANAL. BIOCHEM., 176, 269-277, in an
attempt to transport biomolecules from the solid phase
5   to the gas phase. These methods suffer either from
severe sample decomposition or multiple charging prob-
lems. Other obstacles for mass spectral DNA sequencing
methods include: guaranteeing inadequate mass resolution
at 30,000-200,000 AMU (100-500 base strands); accom-
10 plishing selective and efficient ionization of DNA
strands; and avoiding multiple ionization and/or frag-
mentation of DNA strands.

Laser vaporization may be used for the desorp-
tion of biological molecules into the gas phase, Karas,
15 M. et al., (1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843.
Proteins with molecular weight approaching 175,000
daltons have been molecularly desorbed with this tech-
nique and detected using TOF methods, Karas, M. et al.,
(1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843. Recently,
20 Cotter et al., (1990) RAPID COMMUN. MASS SPECTROM., 4, 99-102
have demonstrated matrix-assisted laser vaporization and
high resolution TOF detection of oligodeoxyribonucleo-
tides with mass up to 1797 Dalton (6 bases). In this
case, the positive molecular ion peak was intense with
25 no apparent strand cleavage. Autoradiographic studies
by Williams et al. suggest that extremely long DNA
strands, containing up to 1,200 nucleotides, (1989)
SCIENCE, 246, 1585-1587, can be transported into the gas
phase intact.
30   Resonance-enhanced multiphoton ionization
(REMPI), has been used to ionize many different biomole-
cules, including nucleotides and nucleosides, Li, L. et
al., (1989) INT. JOURNAL OF MASS SPEC. & ION PROCESSES, 88,
197-210, peptides, amino acids, Grotemyer, J. et al.,
35 (1987) INT. J. MASS SPECTROM. ION PROCESSES, 78, 69-83,
hormones, catecholamines, Pang, H.M. et al., (1988) APPL.

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SPECTROSCOPY, 42, 1200-1206, and purines, Li, L. et al., (1989) INT. JOURNAL OF MASS SPEC. & ION PROCESSES, 88, 197-210.

TOF mass spectrometry has detected proteins with masses approaching 175,000 AMU, Karas, M., Ingendoh, A., Bahr, U., Hillenkamp, F. (1989) BIOMED. & ENV. MASS SPECTROM., 18, 841-843. This would correspond to a DNA strand of approximately 530 bases long. Finally, the extremely high sensitivity a TOF mass spectrometer allows the detection of ultra-low sample amounts in the sub-attomole range.

The difficulties of the prior art are overcome by the methods described herein to analyze an organic sample and/or to determine the base sequence of a nucleic acid.

It is an object of the present invention to use current sequencing technology with a mass spectral method to directly analyze the products of enzymatic DNA sequencing reactions.

It is the object of the present invention to solve inherent problems of the prior art described above using a combination of following techniques: (i) laser vaporization methods to desorb the liquid phase DNA strands into the gas phase; (ii) pulsed molecular beam nozzle techniques to transport the gas phase strands from a flowing helium atmosphere into the vacuum system; (iii) laser ionization methods to resonantly ionize a "tag" molecule on each DNA strand; and (iv) time-of-flight methods for high mass analysis.

It is an object of this invention to desorb biomolecules by mixing the sample of interest in an excess of a "matrix," or chromophore, which is specifically chosen to absorb light where the biomolecule does not. The chromophore absorbs the extremely high powered light (10^6-10^9 watts) that is presented in the short laser pulse (5ns).

It is an object of this invention to use this energy which is deposited in a short time so that all of the matrix and biomolecules are transported into the gas phase before thermal equilibrium can be attained.

5   It is an object of the present invention to place a single positive charge on the vaporized molecules using a technique called resonance-enhanced multiphoton ionization (REMPI). REMPI has been shown to be a very powerful tool for the analytical study of

10   biological materials. The technique is based on selectively exciting an atom or molecule with a laser through specific vibronic states until the ionization energy is surpassed (Fig.2).

        It is an object of the present invention to

15   place on each vaporized DNA molecule a single charge by selective ionization of a covalently attached chromophore or "tag." These charged ions are then detected using time-of-flight (TOF) mass spectrometry.

        It is an object of the present invention to

20   use the combination of a solution-phase laser vaporization method with the ability to measure high masses using a TOF mass spectrometer to provide a rapid (<5 sec) method to completely analyze all of the nested strands produced from a given enzymatic dideoxy sequenc-

25   ing reaction.

        It is a further object of the present invention to determine the sequences of the bases of a nucleic acid sample. Prior techniques are extremely slow and are highly labor intensive.

30   It is also a further object to describe an improved apparatus for passing tagged biological samples from a vaporizing source to an apparatus which would permit the detection of the sequences of the components of a biological sample such as a nucleic acid such as

35   DNA or RNA.

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Summary Of The Invention

Described is a method of analyzing an organic sample, comprising the steps of:

a)   providing an organic sample in a medium
5   which absorbs visible light;

b)   vaporizing the sample and the medium into the gaseous state by subjecting the sample and the medium to electromagnetic radiation from an optical source wherein the vaporizing of the organic sample
10   occurs in a visible light absorbing medium which absorbs the electromagnetic radiation in the visible light region;

c)   ionizing the vaporized sample; and

d)   detecting the contents of the vaporized,
15   ionized sample.

An apparatus is described for accomplishing this analysis comprising an optical source for generating electromagnetic radiation in the visible light region for the purpose of vaporizing the organic sample
20   present in a visible light absorbing medium, an ionization chamber containing an optical source in which the vaporized sample is exposed to electromagnetic radiation that excites the sample and ionizes the sample, and a means to detect the ionized sample to analyze the
25   sample. Most preferably, a mass spectrometer will be used to determine the molecular weights of the ionized sample. In the preferred embodiment, the mass spectrometer is of the time-of-flight type.

To use this apparatus for sequencing DNA or
30   RNA, the sample to be sequenced is used as a template according to the dideoxy sequencing procedure employing in these enzymatic reactions either primers or dideoxy-ribonucleoside 5'-triphosphates that are covalently attached to a chromophore. The four enzymatic reactions
35   products are then subjected individually to vaporiza-

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tion, ionization and mass analysis and the data corre-
lated from each set to generate the sequence for each
nucleic acid sample.

Applicants have discovered that high molecular
5   weight nucleic acid molecules can be vaporized without
fragmentation.   In general, this vaporization is per-
formed by use of a high energy visible laser light in
conjunction with a high concentration of a matrix which
absorbs the laser light energy.   The nucleic acid
10   molecule to be analyzed is mixed with the matrix prior
to vaporization.   When the laser light illuminates the
matrix, the matrix is vaporized and any nucleic acid
molecule within the matrix is entrained, i.e., simulta-
neously vaporized, along with the matrix. By selection
15   of a suitable matrix, wavelength of laser light, and
laser energy level, it is possible to ensure that little
or no fragmentation of the nucleic acid molecule occurs.
The matrix and wavelength of laser light should be such
that the laser light is absorbed by the matrix but not
20   absorbed by the nucleic acid molecule, and chemical
bonds within the nucleic acid molecule are not cleaved.
Applicants have found that use of high laser levels
(above 80 mJ/cm^2, or even 300 mJ/cm^2) for vaporization
produces significantly improved results than use of a
25   lower laser energy.

Brief Description Of The Drawings

FIGURE 1 is a diagrammatic representation of
a dideoxy sequencing method;
FIGURE 2 shows a schematic view of 2-photon
30   resonance enhanced ionization (REMPI) of a molecule M;
FIGURE 3 is a schematic representation of an
apparatus of the present invention;
FIGURE 4 is a schematic representation of a
vaporization chamber of the apparatus in Figure 3;

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FIGURE 5 is a schematic representation of an optical system for vaporization;

FIGURE 6 is an optical system for REMPI;

FIGURE 7 is a side view of an apparatus of the present invention;

FIGURE 8 is a top view of the apparatus of Figure 7;

FIGURE 9 is a spectrum of analyzed samples by use of the apparatus of Figures 7 and 8;

FIGURES 10 and 11 are copies of autoradiographs showing a vaporized oligonucleotide;

FIGURE 12 is a graphical representation of the effect of laser energy on stability of dATP;

FIGURES 13A and 13B are copies of autoradiographs showing the effect of laser energy on populations of DNA molecules; and

FIGURE 14 is a diagrammatic representation of mock data from a mass spectrometer of the present invention, and its analysis to determine a nucleotide sequence. A, T, C and G represent the data obtained from populations of molecules having variable ends terminating in the corresponding nucleotide.

Detailed Description Of The Invention

This invention includes a method for analyzing and identifying the components of an organic sample and an apparatus for accomplishing this method. In the preferred embodiment, the identification is through a determination of the molecular weights of these components and the preferred components are the products of a dideoxy sequencing reaction.

This discovery allows the determination of molecular weights of nucleic acid molecules in a solution or in a solid state, and for the determination of the molecular weights of several different nucleic acid

molecules within a mixture of nucleic acid molecules.
To this end, the invention is particularly useful for
determination of the nucleotide sequence of DNA or RNA,
without the need for any separation of nucleic acid
5   molecules generated in a sequencing reaction.

For example, a population (series) of nucleic
acid molecules can be formed by use of standard tech-
niques, such as those described above, and directly
analyzed by causing vaporization of each of the mole-
10   cules and determination of their molecular weights. If
four such populations of molecules, ending in A, T, G,
or C, respectively, are created, the nucleotide sequence
can be determined by comparing the molecular weights of
molecules within each of the four populations. Such
15   comparisons can be performed by use of a computer, and
allow analysis of a large number of sequencing mixtures
within a very short time.

Because analysis by mass spectrometry does not
require separation of nucleic acid molecules from one
20   another to obtain molecular weight, the methods of this
invention provide significant advantages over prior
sequencing methods which rely, in general, on use of
polyacrylamide gel electrophoresis to separate the
molecules. Thus, the sequencing methods described
25   herein eliminate the most labor-intensive and time-
consuming steps of DNA sequencing methods.

It is also possible, because of the enhanced
sensitivity of this method, to substantially reduce the
amount of reagents needed for sequencing. For example,
30   sequencing can be performed with a reduced amount of a
polynucleotide template and enzyme used in a dideoxy
sequencing reaction. Applicants' method also provides
significant time savings because mass spectrometry
determinations can be made quickly. In addition, the
35   technology described herein can be easily automated for
repeated sample analysis. Thus, the sequencing methods

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described herein have many advantages over currently available methods.

In general, a method of this invention features vaporization of a standard DNA sequencing solution
5   containing nucleic acid molecules of varying molecular weights, modification of the vaporized molecules so that they are susceptible to analysis by mass spectrometry, for example, by ionization, and performance of mass spectrometry on the vaporized and ionized molecules.
10   Thus, in a first aspect, the invention features a method for analyzing a nucleic acid molecule, without fragmenting the molecule, by vaporizing a mixture of the molecule and a matrix by illuminating the mixture with visible laser light absorbed by the matrix
15   and not by the nucleic acid molecule.

By "vaporizing" is meant that the nucleic acid molecule is caused to enter the vapor phase such that it is available for analysis by a mass spectrometer, or available for ionization and subsequent analysis by a
20   mass spectrometer.

By "matrix" is meant any component of a mixture with the nucleic acid molecule which is adapted to absorb visible laser light, and can be vaporized by that laser light, and is adapted to simultaneously cause
25   vaporization of the adjacent nucleic acid molecule. That is, the vaporized matrix entrains the embedded nucleic acid molecule and carries it into the vapor phase. Generally, this matrix is vaporized by visible laser light having a wavelength between about 400 and
30   1100 nanometers, preferably between 500 and 550 nanometers, and most especially 532 nanometers, which is not absorbed by the nucleic acid molecule.

In a related aspect, the invention features a method for determining the nucleotide sequence of a
35   polynucleotide by using mass spectrometry to determine the molecular weights of individual single-stranded

nucleic acid molecules in a population. The population
includes a plurality of single-stranded nucleic acid
molecules generated from the polynucleotide (e.g., by a
sequencing technique described above), each nucleic acid
5   molecule having a different molecular weight and one
defined terminus and one variable terminus which termi-
nates at a specific nucleotide. Generally, this method
is performed without prior separation of the nucleic
acid molecules from each other.

10   By "mass spectrometry" is meant any technique
which allows the molecular weight of a nucleic acid
molecule in the vapor phase to be determined. Those of
ordinary skill in the art will recognize that many
specialized apparatus, generally termed mass spectrome-
15   ters, are known which are specifically adapted to
perform the technique of mass spectrometry, e.g., a
time-of-flight mass spectrometer which is particularly
suited to detection of molecular weight of large mole-
cules.

20   As discussed above, methods for generating
nucleic acid molecules from a polynucleotide to deter-
mine nucleotide sequences are well known in the art.
For example, such techniques include the use of a chain
termination agent in a technique generally called
25   dideoxy chain termination sequencing (see, Sanger et
al., supra, and Tabor and Richardson U.S. Patent No.
4,795,699), or the technique generally described by
Maxam and Gilber, supra. It is particularly important
in the sequencing method that molecules of one molecular
30   weight be provided in approximately equal numbers to
molecules of similar molecular weight as, for example,
described by Tabor and Richardson, U.S. Patent No.
4,962,020. Thus, the population includes several sets
of nucleic acid molecules of identical molecular
35   weights, each set having a different molecular weight
from each other set.

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Each of the nucleic acid molecules in the
population has a defined terminus, that is, each mole-
cule has an identical 3' terminus or 5' terminus con-
taining a chain of identical nucleotides. Each molecule

5   also has a variable terminus, that is, the other of the
3' or 5' terminus is different for each set of nucleic
acid molecules within the population. As will be
recognized by those or ordinary skill in the art, for
each molecule in the population, the variable terminus

10   generally ends in a specific identical nucleotide.

In preferred embodiments of the above aspects,
the determining step includes vaporizing a mixture of
matrix and the population of nucleic acid molecules
derived from the polynucleotide to be analyzed. The

15   mixture is vaporized by illuminating it with visible
laser light absorbed by the matrix. As discussed above,
the nucleic acid molecules are generated by chemical
degradation of the polynucleotide, or by extension of a
short polynucleotide, called a primer, complementary to

20   a portion of the polynucleotide to be sequenced by a DNA
polymerase (e.g., T7 DNA polymerase) in the presence of
a chain terminating agent, e.g., a dideoxynucleoside
triphosphate, preferably in the presence of manganese.
The polynucleotide may be a DNA or RNA molecule.

25   In other preferred embodiments, the determin-
ing step includes comparing the molecular weight of the
nucleic acid molecules, to provide a nucleotide se-
quence, e.g., by use of a computer; the vaporizing step
is performed using a laser adapted to emit a pulse of

30   light, e.g., with a power greater than about 80 mJ/cm^2,
or preferably greater than about 120 mJ/cm^2, or most
preferably greater than about 320 mJ/cm^2; the laser is a
neodium yttrium aluminum garnet laser; and the pulse of
light is less that 10 nanoseconds, and preferably about

35   5 nanoseconds.

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In yet other preferred embodiments, the matrix
is an organic dye, e.g., rhodamine 6G, with a ratio of
matrix weight to total nucleic acid molecule weight
ranging from about 1:1 to 100,000:1, more preferably
5   1,000:1 to 25,000:1; the matrix may also be chosen from
Rhodamine 6G, Rhomdamine 700 or 800, DTTCI, LC8800,
DNTTCI, HDITCI, DDCI4, and dibenzocyanine 45 or from any
one of a number of chromophores which absorb light
between 400-1100 nm; and each nucleic acid molecule is
10   bonded to an ionizable chromophore which allows ioniza-
tion of each molecule, e.g., a fluorescent dye, selected
from the group including fluorescein, rhodamine, tetra-
methylrhodamine, sulforhodamine 101, nitrobenzo-2-oxa-1-
diazaole, anthracene, pyrene, coumarin, acridone, N-5-
15   dimethyl amino naphthene, and derivatives thereof (in-
cluding iodoacetamide, maleimide, isothiocyanate and
succinimidyl carboxylate) and the like. Most prefera-
bly, the chromophore absorbs light of a wavelength
greater than 300 nanometers, and is connected to the
20   molecules by covalent bonding or by a linker arm (see
e.g., Hunkapiller et al., U.S. Patent 4,811,218 and Fung
et al., U.S. Patent 4,855,225), and preferably posi-
tioned between 1 and 50 atoms from the molecule.

Other preferred embodiments further include
25   ionizing the nucleic acid molecules, after the vaporiz-
ing step, and determining the molecular weight of the
vaporized ionized nucleic acid molecules using a mass
spectrometer, e.g., a time-of-flight spectrometer.
Preferably, a single positive charge is placed on each
30   vaporized nucleic acid molecule prior to analysis by
mass spectrometry. One technique for placing such a
single positive charge is by resonance-enhanced multi-
photon ionization (REMPI).

In another related aspect, the invention
35   features an apparatus for determining the nucleotide
sequence of a polynucleotide. The apparatus includes a

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mass spectrometer adapted to determine the molecular
weight of individual single-stranded nucleic acid
molecules (derived from the polynucleotide) in a first
plurality of different populations (e.g., four separate
5   A, T, C, and G populations), each population including
a second plurality of different single-stranded mole-
cules, each having a different molecular weight and one
defined terminus and one variable terminus, the variable
terminus terminating at a specific nucleotide, with the
10   variable terminus of each first plurality of different
populations terminating at a specific nucleotide. Also
provided is a computer adapted to compare the molecular
weights of each of the molecules in the populations to
provide the nucleotide sequence of the polynucleotide.
15   In a further related aspect, the invention
features one or more populations of vaporized and
ionized nucleic acid molecules including a plurality of
different single-stranded nucleic acid molecules each
having a different molecular weight and one defined
20   terminus and one variable terminus, the variable termi-
nus terminating at a specific nucleotide.
             A feature of the invention, which uses laser
desorption of biomolecules, is to mix the sample of
interest into an excess of a "matrix" or chromophore,
25   which is specifically chosen to absorb light where the
biomolecule does not. It is believed that the chromo-
phore absorbs the extremely high-powered light (10^6 - 10^9
watts) that is present in the short laser probe (5 ns).
Possible chromophores includes nicotinic acid, sinapinc
30   acid, ferulic acid, nitrobenzyl alcohol, benzene,
diiodomethane, rhodamine G, and the like. This energy
is deposited in such a short time that probably all of
the matrix biomolecules are transported into the gas
phase before thermal equilibrium can be attained. It
35   has been shown that little or no degradation of thermal-

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ly labile molecules results from this desorption technique.

If the sample does not contain a suitable chromophore for resonance enhanced multiphoton ionization, then a light-absorbing chromophore may be covalently linked to the sample. There are a large number of dye moieties which are appropriate for this ionization process. Suitable dyes include fluorescein and fluorescein derivatives, rhodamine and its derivatives, tetramethylrhodamine and its derivatives, sulforhodamine 101 (Texas red) and its derivatives, nitrobenzo-2-oxa-1-diazole, anthracene, pyrene, coumarin, acridone, N-5-dimethyl amino naphthene, and their derivatives and the like. Derivatives of each of these dyes are commercially available from (Molecular Probes, Inc., Eugene, Or) in forms that can be easily linked to appropriately activated biological or chemical samples (e.g., containing an available amine or thiol group). The most appropriate of the available derivatives of these dyes for the purpose of linking to biomolecules include: iodoacetamide, maleimide, isothiocyanate, and succinimidylcarboxylate and the like. The appropriate functionality on the biomolecule to link to the first two in this series is SH, while the latter two require an NH_2 group present on the biological or chemical sample. For a listing of suitable dyes, see U.S. Patent No. 4,821,218 and 4,855,225, hereby incorporated by reference.

There are a wide variety of standard procedures for chemically binding these types of chromophores to organic and biological molecules (see, for example, U.S. Patent Nos. 4,821,218 and 4,855,225). Amine and thiol-containing nucleic acids can also be prepared and used to link to the appropriate reactive group on the dyes. Oligonucleotides are commercially available from many sources that contain either a reactive amine or

thiol group at the 5' end (Clonetech, Palo Alto, CA).
These are linked to the corresponding reactive groups on
the dyes using standard procedures.   There are also a
number of commercially available oligonucleotides which
5   are coupled to fluorescent dyes.   For example, ABI
(Foster City, CA) sells four oligonucleotides that are
used in their automated DNA sequencer that are covalent-
ly linked to two fluorescein (fluorescein and 2', 7'-
dimethoxy-4,5-dichlorofluorescein) and two rhodamine
10   (tetramethylrhodamine and rhodamine X) derivatives.

              These same dyes can also be covalently linked
to proteins allowing detection and molecular weight
determination of protein mixtures in biological samples.
Unlike the nucleic acid modifications, proteins contain
15   many reactive functional groups which will react with
the preferred linking groups of the dye derivatives
(isothiocyanate, succinimidylcarboxylate, iodoacetamide,
and maleimide).   For example, iodoacetates react predom-
inantly with SH groups of free cysteines but may also
20   react, if cysteines are absent, with methionines,
histidines, or tyrosines. Maleimides are also primarily
thiol-reactive but will also react with a amines at
higher pH. Since many proteins do not have free thiols,
the amine reactive functional groups, isothiocyanates
25   and succinimidylcarboxylate, which react with free
aliphatic amines are often the best choice for these
types of coupling. Essentially, all known proteins have
lysines and most have a free amino terminus. Therefore,
these amine reactive dye derivatives are often the
30   reagents of choice for protein modifications.

Vaporization Chromophore (Matrix)

              It is important in the invention to mix the
nucleic acid molecules to be analyzed with an excess of
a matrix which is specifically chosen to absorb light

energy at a wavelength not absorbed by the molecules. It is believed that the matrix absorbs the extremely high-powered light that is present in a short laser pulse (less than 10 nanoseconds (ns), most preferably

5   less than 5 ns), and is thereby vaporized. Examples of such matrices include Rhodamine 6G, Rhodamine 700 or 800, DTTCI, LC8800, DNTTCI, HDITCI, DDCI-4, and dibenzo-cyanine 45. Applicants believe that, because the laser energy is received by the sample over only a short time,

10 all of the nucleic acid molecules within the matrix are caused to enter the vapor phase by entrainment before any fragmentation of the nucleic acid molecules can occur.

Ionization Chromophore

15   The present invention provides laser vaporization methods to desorb liquid phase nucleic acid molecules into the gas phase, and laser ionization methods to resonantly ionize each nucleic acid molecule. Unmodified nucleic acid molecules are not readily

20 ionized at wavelengths above 300 nm. Thus, an ionizable group is introduced onto each molecule. For example, a single chromophore is introduced into each nucleic acid molecule so that a single positive charge an be intro-duced on each vaporized nucleic acid molecule by reso-

25 nance-enhanced multiphoton ionization (REMPI). For example, referring to Figure 2, an uncharged nucleic acid molecule (M) is raised to an excited electronic state (M*) by a first photon, and then to an ionized state (M+) by a second photon. These ions (M+) can then

30 be detected using time-of-flight (TOF) mass spectrome-try.

          Suitable chromophores or dyes are recited above.

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It is desirable that the chromophore is positioned at least 1 atom and possibly up to 50 atoms away from the nucleic acid molecules strands by use of a linker arm of appropriate lengths.

5   The chromophores can also be covalently linked to a nucleotide. For example, when used for DNA sequencing, these nucleotides can be used as the four dideoxyribonucleotides containing the four bases A, C, G, and T. E.I. DuPont (Wilmington, DE.) sells the four

10   dideoxynucleotides covalently attached to four different fluorescein dyes. ABI sells four dideoxynucleotides covalently attached to four rhodamine chromophores. Also, there are several standard procedures by which a dideoxynucleotide can be coupled to a chromophore. For

15   example, synthesis of the dideoxy-TTP derivative can be accomplished by converting dideoxy-UTP (ddUTP) to 5-(3-amino)allyl ddUTP by the method described by Langer et al. 78 PROC. NATL. ACAD. SCI. U.S.A., 6633, 1981. The cytidine analog can be formed by converting dideoxyuri-

20   dine to the 4-hexylamine derivative using a procedure similar to that described by Horn et al., 17 NUCLEIC ACIDS RES., 6959, 1989, coupling to the NHS ester of a selected chromophore, followed by conversion to the triphosphate by one of several methods, e.g., as described by

25   Kozarich et al., 12 BIOCHEMISTRY, 4458, and Ruth et al., 20 MOL. PHARMOCOL., 415, 1981. The dideoxyadenosine or guanosine derivatives can be similarly prepared by lithiation of the purine ring at C8 followed by alkylation with a suitably protected amine-containing alkyl-

30   halide Barton et al., TETRAHEDRON LETT., 279, 1979. Alternatively, the adenosine derivative can be prepared by iodination at C7 of the adenosine derivative, dide-oxytubercidin, followed by coupling to N-trifluoro-acetylpropargylamine under Pd (0) catalysis. Robins et

35   al., 48 J. ORG. CHEM., 1854, 1983.

It is important that only one chromophore be attached to each nucleic acid molecule to ensure a single positive charge on each molecule after ionization. The chromophore procedures discussed above enable detection of the molecules by allowing the placement of precisely one unit of positive charge per nucleic acid molecule, permitting an exact determination of molecular weight. Single ionization of each molecule greatly simplifies the appearance of a mass spectrum of mixtures of nucleic acid molecules.

It is desirable that the chromophore absorb at a wavelength greater than 300 nm (where DNA weakly absorbs). In addition, the chromophore should have excited states which allow resonant ionization. In one example, when an anthracene chromophore is used, it is believed that the solution phase electron excitations may be centered around approximately 380 nm, 320 nm, and 280 nm. Thus, an ionization scheme as shown in Figure 2 is possible.

Apparatus

The apparatus of the present invention can be used to analyze biological or chemical samples generated from any one of a number of sources. For example, it is possible to analyze blood samples for the presence of various metabolites or proteins or even modified proteins. However, in the preferred embodiment, this instrument will be used to sequence DNA.

The DNA samples to be sequenced are processed according to the Sanger dideoxy sequencing method described above. These reactions are run with the light absorbing chromophore linked to either the primer or the dideoxyribonucleotides. . Each of the four enzymatic reactions containing the dideoxy-terminated product DNA covalently linked to a light-absorbing chromophore are

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then mixed with an excess (e.g., 10-100,000 fold by
weight excess) of rhodamine 6G and each of the four
mixtures are placed individually into sample holder 9.
In the case where the sample is analyzed in the solid
5   phase, the water is removed by evaporation. In the case
where the sample is analyzed as a liquid, the sample
holder is placed directly into the vaporization chamber
21. The sample is then exposed to a 1-100 MW pulse from
the laser at 532 nm which vaporizes the rhodamine 6G,
10   and via entrainment, the biological sample. This
vaporized material is extracted through the pulsed
nozzle 18a by the flowing helium stream, ionized by a 1
MW pulse from the eximer laser, and extracted by the
three kilovolt potential applied to the 90% transmission
15   grid into the time-of-mass spectrometer. The molecular
weights of the ions detected by the mass spectrometer
are recorded. Then this entire process is repeated in
sequence for the remaining three dideoxy sequencing
reactions, the results from the four samples correlated,
20   and the DNA sequence deduced as is done with manual or
other automated sequencing methods.

          Referring to Figure 3, a schematic representa-
tion of an apparatus of the invention is provided.
Specifically, apparatus 8 includes a sample holder 10 in
25   which the nucleic acid molecule of interest is placed
within a matrix. This sample holder is positioned to
allow electromagnetic radiation, e.g. laser light, from
a vaporization laser 14 to contact the nucleic acid
molecule. Also provided is a pulsed nozzle 18A which is
30   positioned relative to sample holder 10 to allow vapor-
ized matrix and nucleic acid molecule to pass in a
series of pulses as a desorbed sample (shown generally
at 16) through a pair of beam skimmers 17 into a loca-
tion 15 at which the volatilized nucleic acid molecules
35   can be ionized by electromagnetic radiation from an
ionization laser 32. Such ionized and vaporized nucleic

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acid molecules are then directed by use of a high voltage electric field 36 into a mass spectrometer (shown generally as 34) through an ion projectory 38 via an ion reflector 40 to a multichannel detector 42.

5   Referring to Figure 4, a detail of a vaporization chamber used in apparatus 8 (shown in Figure 3) is provided. This chamber includes the sample holder 10 on which aqueous sample 12 is deposited. Above this holder is a pulsed nozzle 18A which extracts gaseous sample

10   resulting from vaporization of the sample by laser light from vaporization laser 14, together with the carrier gas, helium, which enters vaporization chamber 21 through inlets 19 and 19A. The carrier gas causes the vaporized sample to pass out of the vaporization chamber

15   toward the laser beam of ionization laser 32 where the sample is ionized. A window 20 is provided in the upper portion of vaporization chamber 21, and a second transparent window 29 is provided in the lower portion of the chamber, to allow passage of laser light from vaporiza-

20   tion laser light 14. The base of the pulse nozzle is shown generally by the numeral 26.

       The vaporization chamber 21 shown in Figure 4 can be maintained at either vacuum or ambient pressure. While not specifically shown, a high efficiency pumping

25   system is provided to evacuate the low pressure chambers of this apparatus. Generally, the vaporization chamber is maintained at ambient pressure. When the sample is a liquid, the sample holder is oriented in a horizontal plane so that a liquid sample will not drip or run, and

30   is situated so that it can be exposed to electromagnetic radiation. The sample holder is constructed from either polished 305 stainless steel or glass and is removable. It is affixed so that the sample can be reproducibly positioned in precisely the same location with respect

35   to the pulsed nozzle. Alternatively, the sample can be dried to a solid by evacuation.

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The source of the electromagnetic radiation to
cause vaporization is generally a laser, e.g., a neodi-
nium yttrium aluminum garnet (Nd YAG) laser. This laser
is operated in a mode which provides radiation at 532 nm
5   up to 500 mJ/cm^2. The light is columinated to provide
high enough power to effect vaporization of at least a
portion of the sample. Figure 5 shows that the columi-
nated light 14' from a laser is directed through an
optical system. This system includes two pellin broca
10   prisms (not shown) which remove unwanted frequencies of
light to a beam dump for removal. The remaining light
is then directed, first through an iris to restrict beam
size, and then through focusing optics 50 constructed
from a non-absorbing quartz S1 UV optical flat (1" x
15   1/8") into the vaporization cell to impinge onto the
sample from above. The laser is operated in such a
manner so as to produce an intense flash of electromag-
netic radiation which will be absorbed by the vaporiza-
tion matrix and not the sample.
20   Once vaporized, as discussed briefly above,
the sample is ejected into a helium atmosphere for
subsequent processing. The helium injection and exhaust
ports are positioned to maximize the flow of vaporized
product toward the pulsed nozzle. The flow of helium is
25   maintained using a He flow meter as supplied from Tylan
Corporation. The output of the helium flow is connected
to a pump via a metering valve. The pulsed valve is
commercially available from Thermionics Laboratory, beam
dynamics. The pulsed valve is positioned between 1 and
30   10 mm above the surface of the sample to be vaporized.
The pulsed valve is timed to open 0.1 to 100 microsec-
onds after the firing of the vaporization laser. The
sample is extracted from the vaporization chamber into
the ionization chamber, and a potential simultaneously
35   applied to a 905 transmission grid to extract any ions

which form during the vaporization process, and survive
the transit through the pulsed nozzle.

Once the sample has entered the vacuum cham-
ber, a second intense burst of electromagnetic radiation
5   is delivered into the apparatus from an excimer or YAG
laser source. In the preferred embodiment, the ionizing
radiation is at 300-500 nm, most preferably 300-380,
e.g., 345 nm. As shown in Figure 6, this beam is
developed using optical components. The beam 32' is
10   directed through a telescoping cylindrical lens 52 to
shape the beam into a compact pulse. The beam is then
passed through slits 54 to define the final shape of a
ribbon of about 1 mm x 5 mm in size. The beam is then
directed through a quartz window 56 into the vacuum
15   chamber. In the vacuum chamber, the light intersects
the beam of sample and matrix molecules at a 90 degree
angle. The sample absorbs the radiation and the con-
tained molecules ionized by resonant multiphoton pro-
cesses.

20   The ionized sample is extracted by a three
kilovolt potential applied to a 90% transmission grid
(see Figure 3, field 36) which is 10 cm from the plane
of the ionizing radiation. The ions formed in the
electromagnetic pulse are then extracted into a mass
25   spectrometer 34 (Figure 3), e.g., a time-of-flight mass
spectrometer, such as a Bruker TOF 1 (Bruker Instru-
ments, Inc. of Billerica, Mass.) This system operates
at 30 Hz, with a very large sample depth (128K) and high
resolution (16 bit). This system includes the required
30   electronic controls and an ultra high vacuum pumping
system that can be used for the ionization chamber.

Methods

The polynucleotide to be sequenced is pro-
cessed according to the Sanger dideoxy sequencing

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reaction method described above, or any other sequencing method. These reactions are preferably run with a light absorbing chromophore linked to either the primer or the dideoxyribonucleotides.   Each of the four enzymatic
5   reactions containing the dideoxy-terminated molecule covalently linked to a light-absorbing chromophore is then mixed with an excess (e.g., 10-100,000 fold molar excess) of rhodamine 6G, and each of the four mixtures placed individually into the sample holder. When the
10   sample is in the solid phase, the surrounding liquid medium, e.g. water, is removed by evaporation. When the sample is a liquid, the sample holder is placed directly into the vaporization chamber.

The sample is exposed to a 1-100 MW pulse from
15   the laser at 532 nm which vaporizes the rhodamine 6G, and via entrainment, the nucleic acid molecule. This vaporized material is extracted through the pulsed nozzle by the helium stream, ionized by a 1 MW pulse from the excimer laser, and extracted by the three
20   kilovolt potential applied to the 90% transmission grid into the time-of flight mass spectrometer. The molecular weights of the ions detected by the mass spectrometer are recorded. This process is then repeated in sequence for the remaining three dideoxy sequencing
25   reactions, the results from the four samples are correlated, and the nucleotide sequence deduced. An example of such data is shown in Figure 14. Correlation of these data can be performed manually or by computer using a program which determines the relative molecular
30   weights of each molecule in each population of molecules. Such a program is readily formulated by those skilled in the art.

Those skilled in the art will understand that there are many variations of the above apparatus and
35   method which fall within the purview of this invention. For example, different matrices can be used for the

vaporization processes and different chromophores can be
used for the ionization processes, as can different
sources of electromagnetic radiation be used.   The
sample may require pre-treatment by various procedures
5   to increase sensitivity levels, for example, removing
the template polynucleotide, removing the substrate
nucleotides, exchanging counterions, or removing any
proteins prior to analysis.   Furthermore, the sample
holder and vaporization chamber can be modified so as to
10   accept multiple samples by the addition of a movable
stage that will bring each of the various samples into
register for the vaporization step.
              The "tag" molecule in this mass spectral
technique will perform a role analogous to the fluores-
15   cent tag employed in other automated electrophoretic DNA
sequencing techniques.   In the mass spectral technique,
the "tag" will enable the detection of the DNA compo-
nents by allowing the placement of precisely one unit of
positive charge per DNA strand, permitting an exact
20   determination of molecular weight or strand length.
Single ionization of each strand will greatly simplify
the appearance of mass spectrum of mixtures of DNA
strands.
              For a desirable REMPI tag for mass spectral
25   analysis of DNA strand, it is most desirable that the
molecule have an adsorption band greater than 300 nm
(where DNA weakly absorbs).   Secondly, the molecule
should have excited states which allow resonant ioniza-
tion.   Lastly, the molecule should be chemically attach-
30   able to the DNA strand during the enzymatic reaction.
Utilizing a tetramethylrhodamine chromophore, it is
believed that the solution phase electron excitations
may be centered around approximately 350 nm, 250 nm, and
200 nm for similar compounds.   It is also desirable that
35   the tag dye molecule be positioned at least 8 atoms and
possible up to 29 atoms away from the DNA strands,

depending upon the length of the linker arm that is chosen.

The ionized sample is then extracted by a three kilovolt potential applied to a 95% transmission grid which is 10 cm from the plane of the ionizing radiation. The ions formed in the electromagnetic pulse are then extracted into a mass spectrometer. In the preferred embodiment, the mass analysis system is of the time-of-flight type, such as a Bruker TOF 1 (Bruker Instruments, Inc. of Billerica, Mass). This system has been developed to operate at 30 Hz, with a very large sample depth (128K) and high resolution (16 bit). This system is complete in terms of control electronics and also contains all of the ultra high vacuum pumping systems that will be required for the ionization chamber.

The following examples are illustrative of the invention. They were performed in the sequencing chamber shown in Figures 7 and 8. The chamber consists of a solid film vaporization system juxtaposed to a REMPI TOF chamber. With the vaporization system, a 2 μl spot of a mixture of sample and rhodamine 6G is placed on a glass microscope slide, the sample allowed to dry and the glass slide fixed to a stainless steel rod sample holder. The spot is irradiated with a laser pulse and the vapor plume travels toward the electro-static grid plates of the time-of-flight mass spectrometer.

The lens system for the mass spectrometer is shown in Figure 7. Four grid plates (1-4) form the acceleration optics. The following is a description of the ion optical system starting from the leftmost grid plate shown in Figure 7. The first plate has a 1/8" aperture in its center. This plate is grounded for these examples. The next plate contains a 90% transmission grid which is typically biased positive 1000 volts

with respect to ground. One function of this grid is to repel positively charged ions back to the ground plate so that only neutral molecules enter the ionization region of the mass spectrometer. The next plate is

5   typically biased positively to 950 volts with respect to ground. The region between plates 2 and 3 is called the ionization region because the resonance-enhanced multi-photon ionization occurs here. This region also forms a low voltage extraction region for the dual slope

10 acceleration scheme which serves to decrease the full-width-at-half-maximum of the signal peaks. The fourth plate contains an 82% transmission grid and is grounded. The region between plates 3 and 4 represents an acceler-ation of 950 eV per ion. The ions then enter a 63cm

15 field free drift region of the mass spectrometer.

         At the end of the mass spectrometer is a high molecular weight ion detector 68. This consists of one stage from a CuBe electron multiplier detector. This "venetian" blind ion conversion stage is biased to 10

20 keV to convert high molecular weight ions to smaller ions and electrons. This type of ion conversion system has the added advantage of protecting the multichannel ion detector from carbonaceous contamination. The resulting particles are then directed to the micro-

25 channel plate detector for current amplification. The signal is amplified further in a fast ampli-fier/discriminator. The signal is then directed to a digital storage scope where current or signal is stored as a function of time. A plot of current versus time

30 forms the time-of-flight mass spectrum.

         A schematic of the entire sequencing system is shown in Figure 8. The vaporization chamber and TOF mass spectrometer are pumped by a turbo molecular vacuum pump. The laser beams necessary for ionization and

35 vaporization enter through quartz windows. The optical setup allows the beams to enter the chamber through the

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same window. The vaporization laser is directed at the front of the thin film system by a turning prism, mounted as shown in Figure 7. The ionization beam travels parallel to the plane of the extraction plates.

5   The timing between the two laser pulses is maintained by precision delay circuits. The minimum vacuum for an experiment is approximately 1×10^{-4} torr.

The DNA sample mixture 74 was spotted onto a glass coverslip attached to the end of the stainless

10   steel sample positioner. The mixture of laser dye and tagged DNA sample was allowed to dry into a solid thin film on the coverslip. The sample was then loaded into the vacuum chamber and pumped to a pressure of approximately 5×10^{-6} torr.

15   In both example 1 and 2 described below, the vaporization laser struck the thin film at an angle of approximately 45 degrees from the surface normal. The vaporization laser used was the second harmonic of a Nd YAG III laser, (532 nm, 6 ns pulse length, variable

20   power). The YAG laser was equipped with gaussian optics so that the photon density within the beam was approximately constant across the diameter of the beam. The diameter of the vaporization laser beam was irised to 1 mm. The fluence of the beam ranged between 10 and 80

25   mJ/cm^2 s measured by a power meter (not shown). The sample positioning system was rotatable so that fresh sample could be continuously brought into the area of vaporization, if necessary.

A multiphoton ionization laser 80 was used to

30   generate a 345-370 nm photon and 15 ns pulse length, which passed parallel to the plane of the thin film at a distance of 11 mm from the surface. The beam was irised and passed through 1 mm slits. The beam shape of this ionization laser was a ribbon having dimensions 1

35   mm x 7 mm. for the experiments requiring ionization of the DNA sample, the excimer laser was triggered to fire

at a time of 45 microseconds after the vaporization laser hit the target. The pulse of photons from the ionization laser was detected using a photodiode. The signal from the photodiode defined time = zero for the
5   time-of-flight measurement.

Example 1

A sample of anthracene-labeled thymidine-5'-monotriphosphate was mixed at a 1 to 1 molar ratio with rhodamine 6G dye. The sample was then spotted onto the
10 sample holder as described previously. The vaporization laser at 532 nm was directed onto the sample thin film with a fluence of 40 mJ/cm^2. The ionization laser at 345 nm was maintained at 30 mJ/cm^2. A representative time-of-flight spectrum prepared from the oscilloscope 86
15 connected to the multichannel detector inputs 68 and the photodiode 82 via lines 88 and 90, respectively, is shown in Figure 9.

In this plot (Figure 9), the current signal output from the multichannel detector was plotted as a
20 function of time in microseconds. The peak appearing at 32.7 microseconds, labeled A, was identified as pure rhodamine 6G (MW = 479 AMU) by control experiments where no labeled nucleotide was present.

When the anthracene-labeled nucleotide sample
25 was added to the thin film, the peak at 41 microseconds, labeled B, appeared. This arrival time implied that a species of approximately 650 AMU was present in the vaporized sample. Therefore, the mass calculated for the tagged nucleotide is 650 AMU.

30 ## Example 2

In this example, the ion detection system of Figure 7 was removed from the apparatus and a piece of

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filter paper installed to collect all of the laser
vaporization materials. A 4 mm diameter hole was placed
in the filter paper to allow passage of the vaporization
laser. The paper was positioned at a distance of 10 mm
5   from the vaporization spot on the thin film.

An oligonucleotide (50 pmol) having the
sequence 5'-GTTTTCCCAGTCACGAC-3' was synthesized,
purified by high pressure liquid chromatography (HPLC),
and labeled at the 5' end with ^{32}P using polynucleotide
10   kinase. The labeled oligonucleotide was purified from
unreacted ATP using a Waters Associates Sep-Pak C18
cartridge (Maniatis et al., Molecular Cloning, A Cloning
Manual), Cold Spring Harbor Press, New York, 1982). The
final specific activity of the oligonucleotide was 300
15   counts per minute per femtomole (cpm/fmol). Five
picomoles (pmol) of the oligonucleotide was dissolved in
4 μl of water containing 10 mg/ml rhodamine 6G. This
resulted in a final molar ratio of sample to matrix of
1:17,000. This mixture was then spotted in two 2 μl
20   aliquots on a glass cover slip. The dried sample was
placed in the sample chamber, the chamber evacuated to
5 x 10^{-6} torr, and then the sample was exposed to the
second harmonic of the Nd YAG III laser (532 nm, 8 ns
pulse length) at a power equal to 130 mJ/cm^2. The filter
25   containing the putative vaporized DNA was removed and
the process twice repeated on fresh samples at power
levels equal to 85 and 45 mJ/cm^2, respectively. Each
filter was then exposed to Kodak XAR-5 X-ray film to
obtain the distributions shown in Figure 10: panel A,
30   130 mJ/cm^2; panel B, 85 mJ/cm^2; panel C, 45 mJ/cm^2.

Three features of the vaporization process are
revealed by this analysis. First, as the laser power
was increased, the amount of molecular vaporization
product also increased. Second, the images of the
35   vaporized material on the filter paper revealed a highly
directional vaporization process. The distribution is

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peaked in the normal direction and was considerably tighter than a simple cosine distribution expected for a thermal desorption process. Third, the ^{32}P present on the filter paper was evenly distributed, as is expected for molecular vaporization. Spallation, or the removal of macroscopic pieces of the mixture, has been shown in prior studies (Nelson et al., SCIENCE 1585, 1989) to lead to a spotted or speckled appearance. Spallation features, were observed in our experiment when vaporization was performed at atmospheric pressure on a liquid sample. Taken together, the images of the distribution obtained in these experiments strongly suggest that individual molecules were being vaporized.

Example 3

To characterize the products obtained from the laser vaporization of the above 17-mer, the radioactive material on each filter (Example 2) was eluted by soaking in water, and each mixture then analyzed by polyacrylamide gel electrophoresis.

The autoradiographs of the filter papers shown in Figure 10 were used to indicate where the vaporized radioactive oligonucleotide was deposited onto the filters. These portions were excised and then extracted with two 150 μl portions of water. The resulting solutions were concentrated, and loaded onto a 20% polyacrylamide gel: (Figure 11) lane 1, 130 mJ/cm^2; lane 2, 85 mJ/cm^2; lane 3, 45 mJ/cm^2; lane 4, starting oligonucleotide. The gel was electrophoresed at 1000 V for 2 hr, and the positions of the bands determined by autoradiography. The positions of inorganic phosphate and nucleotide were determined by running authentic samples in adjacent lanes.

The sample vaporized using a laser power of 45 mJ/cm^2 showed extensive strand scission, giving rise to

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nucleic acid molecules having an average chain length of
four nucleotides (Figure 11, lane 3; cf. starting sample
in lane 4).   Applicants believe that strand breakage
occurred through activation of the phospodiester bond.
5   Also, a great deal of the label was observed as free
inorganic phosphate (P_i). However, the samples vaporized
at 130 and 80 mJ/cm^2 (Figure 11, lanes 1 and 2) displayed
no observable strand scission, although substantial
amounts of phosphate were produced. There are several
10   possible explanations for this observation. First, it
is most likely that at lower laser powers the molecules
remain in the thin film longer, causing the desorption
process to mimic thermal decomposition, resulting in a
much higher probability that bonds will be broken.
15   Conversely, the vaporization process becomes a non-
thermal, non-equilibrium photochemical process at high
laser fluences. Second, the vaporized product should be
less decomposed at high fluences because the density of
desorbed material increases as the laser power increase.
20   As the density increases, the number of cooling colli-
sions increase and the vaporization process resembles a
free jet expansion. A third possibility is that as the
fluence of the vaporization beam increases, new elec-
tronic states in the desorbed rhodamine 6G are accessed
25   via multiphoton absorption which serve to more effi-
ciently transfer the nucleic acid molecules into the gas
phase. This is supported by the fact that at the higher
laser powers almost none of the laser dye travels to the
filter paper without decomposition, whereas at lower
30   incident fluxes much of the laser dye arrives intact.

Example 4

To more fully characterize the bond-breaking
process observed in the 17-mer experiment, the vapori-
zation of $[\alpha-^{32}P]$ dATP as a function of laser power was

studied.   [α^{-32}-P] ATP (60 pmol, 3000 Ci/mmol) was dissolved in 20 μl of water containing 10% methanol and 10 mg/ml rhodamine 6G. Sample spots were prepared and vaporized as described above in Example 2 at powers

5   equal to 320, 208, 180, 129, 85 and 45 mJ/cm^2 using a fresh spot for each power level. The resulting filters were processed as described above in Example 3, and the concentrated solutions adjusted so that each had 20,000 cpm/μl. Two microliters of each solution containing the

10 materials which had been vaporized were then eluted from the filter papers, spotted onto a glass PEI-Cellulose F TLC plate (EM Science), and eluted with a solution of 0.6 M LiCl in 1.0 M formic acid. The plate was dried, and the amount of radioactivity present in each spot

15 determined using an Ambis Radioactivity Image Scanner. The identity of the analyzed components was determined by co-spotting with authentic samples.

            Referring to Figure 12, each point represents the average of at least two determinations from two TLC

20 analyses.   (O) dATP, (●) dAMP, (▢) pyrophosphate, and (▉) phosphate. This analysis indicates that up to five species are observed in the vaporized sample, depending on the vaporization condition. As predicted from the oligonucleotide experiments described in Example 3, the

25 highest power levels result in little decomposition of the vaporized product. At 320 mJ/cm^2, approximately 90% of the radioactivity present on the filter paper was recovered as dATP. What little degradation that occurred resulted in the formation of dAMP and inorganic

30 phosphates (mono-, diand tri-phosphates). As the laser power was reduced, several trends become evident: (i) less dATP survived the laser vaporization; (ii) less tripolyphosphate was observed (not shown; less than 4% was produced at even the highest power level); (iii)

35 more phosphate and diphosphate were formed; and (iv) more dAMP was produced. The production of the inorganic

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phosphate is consistent with photochemical activation of the P-O-P bonds at intermediate vaporization power. The incident photon fluence was high enough that this activation proceeded through a two-photon excitation of
5   the 274 num electronic absorption band in the phosphate group.   At the highest powers, the P-O-P bonds were still activated, but the energy was most likely quenched by collisional deactivation. At low vaporization power, the desorption mechanism became more thermal in nature.
10   As the molecules received thermal energy, the weakest bond, the phosphodiester bond, was expected to, and observed to, break.

Example 5

The fact that efficient molecular vaporization
15   with very little bond breakage occurred at laser powers over 300 mJ/cm^2 suggested that it might be possible to effect the molecular vaporization of very long DNA strands.   To test this prediction, two dideoxy DNA sequencing reactions were performed under conditions
20   where the average chain length produced was either approximately 65 or approximately 400 nucleotides long. Two DNA sequencing reactions were carried out using SEQUENASE™ T7 DNA polymerase (United States Biochemical, Cleveland, OH) under conditions where the short (Figure
25   13, panel A) or long (Figure 13, panel B) DNA sequences were synthesized as described by the manufacturer.

For the reaction used to prepare short dideoxy C terminated DNA fragments (Figure 13, panel A), a 50 μl labeling reaction was prepared containing M13mp18 DNA,
30   [α-^{32}P] dATP and Mn^{2+} buffer, using the manufacturer-recommended protocol.   Immediately prior to vaporization, the DNA was denatured and mixed with rhodamine 6G. This mixture (2μl) was spotted onto a glass cover slip. The sample was vaporized and the filters processed, as

described above in Examples 2 and 3. As was found in Example 2, with the vaporization of the nucleotide and oligonucleotide, the pattern of the autoradiograms revealed virtually no evidence for spallation (not

5   shown). The ^{32}p-labeled materials were then eluted from the filters, and run on a high resolution polyacrylamide sequencing gel (Figure 13). The concentrated solutions were loaded onto a denaturing 8% polyacrylamide gel and electrophoresed for 2 hr at 55 watts: Figure 13, panel

10 A, lane 1, 0.02 μl of the starting sequencing reaction prior to vaporization; panel A, lanes 2-5, samples recovered following vaporization at 320, 260, 210, and 160 mJ/cm^2, respectively.

The reaction used to prepare the long DNA

15 sequences was virtually identical except that the Mn^{2+} buffer was not used and the termination mix contained a 3:2 ratio of normal dideoxy C termination mix and extension mix. Figure 13, Panel B, lanes 1-4, correspond to samples recovered following vaporization at 320, 260,

20 210, and 160 mJ/cm^2, respectively; panel B, lane 5 contained 0.006 μl of the starting sequencing reaction prior to vaporization. For both panels A and B, standard G, A, T, and C sequencing reactions were run in parallel in order to precisely determine the lengths of

25 the indicated bands.

It is evident from this analysis that extremely large DNA molecules can be efficiently vaporized without any noticeable strand cleavage or degradation. In the case of the sequencing reaction containing

30 products having an average length of 65 nucleotides (Figure 13A), bands up to 85 nucleotides in length were visible. Longer exposures (not shown) indicated the presence of longer strands (in the 120 to 140 nucleotide range). The banding pattern for the samples generated

35 at each of the laser powers (Figure 13A, lanes 2-5) was as sharp as the starting material (Figure 13A, lane 1),

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strongly suggesting that no strand degradation was
occurring. However, unlike the 17-mer experiment, we
cannot rule out the possibility of trace amounts of
strand scission at random position on these long
5   strands. The intensity distributions of the vaporized
samples were substantially different than that of
starting sample. For example, the ratios of the 20-mer
to 75-mer were compared for the two samples by densito-
metric scanning of the autoradiograms. The results
10   indicated that the intensity of the 75-mer bands were
reduced in relative intensity by 90% for the vaporized
sample. This latter point is further strong support for
molecular vaporization, since spallation would be
expected to generate materials on the filters having
15   bands intensity distributions identical to the starting
samples. Similar results are obtained from the sequenc-
ing reaction carried out to give very long labeled DNA
strands (Figure 13B). Careful analysis of this gel
revealed that DNA strands in excess of 1000 nucleotides
20   long had been vaporized.

The sequencing method described here has
numerous advantages over currently available or proposed
approaches. First, it does not require that DNA se-
quencing products be run on a polyacrylamide gel. This
25   component of manual or automated DNA sequencing is the
most labor-intensive and time-consuming portion of DNA
sequencing. Second, this method does not require the
use of a radioisotope. Both this and the prior point
should significantly reduce the expense currently
30   associated with sequencing by reducing labor, chemical,
and disposal costs. Also, the throughput that can be
expected for this instrument may be 1000 times, or more,
that obtained from currently available automated sequen-
cers. An important consideration is that the technology
35   described here can be easily automated for repeated
sample analysis. Since the mass analysis can be ob-

tained very rapidly, a conservative estimate for the time involved in generating a single DNA sequence is certainly less than one minute, and should be as little as a few seconds. Thus, an instrument running continu-
5   ously might conceivably be able to sequence well above a million bases per day.

The apparatus described herein has not been used previously to make determinations of the molecular weights of biological samples. Because of the unique-
10 ness of the apparatus, unique materials are prepared by virtue of the ionization occurring by the ionization laser after the DNA sample has been vaporized. It is because of this unique difference that the ionized molecules have enhanced capability of being detected
15 more accurately and sensitively through the mass spec equipment. It is believed that these ionized molecules have not been described previously.

While the forms of the invention herein described constitute presently preferred embodiments,
20 many others are possible. It is not intended herein to mention all of the possible equivalent forms or ramifi-cations of the invention. It is understood that the terms used herein are merely descriptive rather than limiting, and that various changes may be made without
25 departing from the spirit or scope of the invention. Other embodiments are within the following claims.

What Is Claimed Is:

1. A method analyzing an organic sample, comprising the steps of:

a) providing an organic sample in a medium which absorbs visible light;

b) vaporizing the sample and the medium into the gaseous phase by subjecting the sample and the medium to electromagnetic radiation from an optical source wherein the vaporizing of the organic sample occurs in a visible light absorbing medium which absorbs the electromagnetic radiation in the visible light region;

c) ionizing the vaporized sample; and

d) detecting the contents of the vaporized, ionized sample.

2. The method of claim 1 wherein the organic sample to be analyzed has an ionizable chromophore covalently bonded to the sample.

3. The method of claim 1 wherein the light absorbing medium is an organic dye.

4. The method of claim 1 wherein the light absorbing medium is in the liquid state.

5. The method of claim 1 wherein the light absorbing medium is in the solid state.

6. The method of claim 1 wherein the sample to be detected is selected from the group consisting of a saccharide, a polysaccharide, a lipid, a hormone, a neurotransmitter, a heme, a nucleotide, a nucleoside, a metabolite, and a peptide.

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7. The method of claim 1 wherein the sample is a protein.

8. The method of claim 1 wherein the sample is a nucleic acid.

5   9. The method of claim 1 wherein the light absorbing medium has the ability to absorb light at a wavelength greater than 500 nanometers.

10. The method of claim 1 wherein the light absorbing medium is comprised of a dye.

10   11. The method of claim 2 wherein the chromo- phore to be bonded to the sample is a dye.

12. The method of claim 2 wherein the chromo- phore to be bonded to the sample is a fluorescent dye.

13. A method for analyzing a nucleic acid
15 molecule comprising the step of vaporizing a mixture of said nucleic acid molecule and a matrix by illuminating said mixture with visible laser light absorbed by said matrix.

14. A method for determining the nucleotide
20 sequence of a polynucleotide comprising the step of determining, by mass spectrometry, the relative molecu- lar weights of single-stranded nucleic acid molecules in a population comprising a plurality of single-stranded nucleic acid molecules, each molecule having a different
25 molecular weight and one defined terminus and one variable terminus, said variable terminus terminating at a specific nucleotide, wherein said molecules are generated from said polynucleotide.

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15. The method of claim 14 wherein said determining step is performed without prior separation of each of said molecules from each other.

16. The method of claim 14 wherein said determining step comprises vaporizing a mixture of said population and said matrix by illuminating said mixture with visible laser light absorbed by said matrix.

17. The method of claim 14 wherein said nucleic acid molecules are generated by chemical degradation of said polynucleotide.

18. The method of claim 14 wherein said nucleic acid molecules are generated by extension of a primer, complementary to a portion of said polynucleotide, by a DNA polymerase in the presence of a chain terminating agent.

19. The method of claim 18 wherein said chain terminating agent is a dideoxynucleotide triphosphate.

20. The method of claim 18 wherein said DNA polymerase is T7 DNA polymerase.

21. The method of claim 18 wherein said extension is performed in the presence of manganese.

22. The method of claim 14 wherein said polynucleotide is DNA molecule.

23. The method of claim 14 wherein said polynucleotide is an RNA molecule.

24. The method of claim 14 wherein said determining step further comprises the step of comparing

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molecular weights of said nucleic acid molecules gener-
ated from said polypeptide to provide said nucleotide
sequence.

25.   The method of claim 24 wherein said
comparing step is performed using a computer.

26.   The method of claim 1, 13 or 16 wherein
said vaporizing step is performed using a laser adapted
to emit a pulse of light.

27.   The method of claim 26 wherein said laser
is adapted to emit a pulse of light having a duration of
less that 10 nanoseconds.

28.   The method of claim 27 wherein the power
of said laser light is greater than about 80 mJ/cm^2.

29.   The method of claim 26 wherein said laser
is a neodium yttrium aluminum garnet laser.

30.   The method of claim 13 or 16 wherein said
illumination is at a wavelength of from about 400 to
about 1100 nm.

31.   The method of claim 13 or 16 wherein said
matrix is an organic dye.

32.   The method of claim 31 wherein said
organic dye is rhodamine 6G.

33.   The method of claim 13 or 16 wherein said
matrix is selected from the group consisting of Rhoda-
mine 6G, Rhodamine 700 or 800, DTTCI, LC8800, DNTTCI,
HDITCI, DDCI-4, and dibenzocyanine 45.

-45-

34. The method of claim 13 or 16 wherein said matrix has the ability to absorb light at a wavelength of from about 400 nm to about 1100 nm.

35. The method of claim 13 or 16 wherein said matrix absorbs light at a wavelength at which DNA does not absorb light.

36. The method of claim 13 or 16 wherein said matrix absorbs light at a wavelength at which RNA does not absorb light.

37. The method of claim 13 or 16 wherein said vaporizing step occurs without fragmenting said polynucleotide.

38. The method of claim 1, 13 or 16 wherein the molar ratio of said light absorbing matrix to said polynucleotide is from about 1:1 to about 100,000:1.

39. The method of claim 13 or 16 wherein a said nucleic acid molecule has a single positive charge.

40. The method of claim 13 or 16 wherein a said nucleic acid molecule is bound to an ionizable chromophore.

41. The method of claim 40 wherein a said nucleic acid molecule is chemically bound to said chromophore by a linker arm.

42. The method of claim 2 or 40 wherein said chromophore is selected from a group consisting of fluorescein, rhodamine, tetramethylrhodamine, sulfor-hodamine, nitrobenzyl-2-oxa-1-diazole, anthracene,

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pyrene, coumarin, acridone, N-5-dimethylaminonaphthene, and derivatives thereof.

43. The method of claim 42 wherein the derivatives are selected from the group consisting of iodoacetamide, maleimide, isothiocyanate and succinimidyl carboxylate.

44. The method of claim 13 or 16 further comprising the step of ionizing said nucleic acid molecules after said vaporizing step.

45. A method according to claim 1 or 44 wherein said ionizing is performed using a laser adapted to emitting a pulse of light.

46. The method of claim 45 wherein said laser is adapted to emit a pulse of light of less than 20 nanoseconds.

47. The method of claim 44 further comprising the step of determining the molecular weight of any vaporized, ionized nucleic acid molecule.

48. The method of claim 1 or 47 wherein said molecular weight is determined using a mass spectrometer.

49. The method of claim 48 wherein said mass spectrometer is a time-of-flight mass spectrometer.

50. The method of claim 14 wherein the molecular weights of said molecules in a plurality of said populations are determined, said molecules in each said population having a variable terminus terminating

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at a different specific nucleotide from other said
populations.

51. The method of claim 50 wherein said
method further includes comparing the molecular weights
5   of said molecules with a computer.

52. A method for determining the base se-
quence of a nucleic acid strand wherein four separate
nucleic acid samples derived from the strand are frag-
mented such that each fragment in a particular sample
10   terminates at one of the bases adenosine (A), cytosine
(C), guanosine (G), or thymidine (T), comprising the
steps of:
          (a) providing an organic sample comprised of
the four samples of the nucleic acid in a light absorb-
15   ing medium which absorbs light;
          (b) vaporizing the sample and the light
absorbing medium into the gaseous state;
          (c) ionizing the vaporized sample; and
          (d) detecting the contents of the vaporized,
20   ionized sample.

53. An apparatus to analyze an organic sample
present in a visible light absorbing medium comprising:
          an optical source for generating electromag-
netic radiation in the visible light region capable of
25   vaporizing the organic sample in the visible light
absorbing medium which absorbs visible light;
          a source of ionizing radiation capable of
ionizing the vaporized sample; and
          an apparatus, juxtaposed to the vaporized
30   ionized sample capable of determining the contents of
the vaporized, ionized sample.

54. The apparatus of claim 53 wherein the apparatus capable of determining the contents of the vaporized ionized sample is a mass spectrometer.

5   55. The apparatus of claim 53 wherein the source of vaporizing radiation is a laser.

56. The apparatus of claim 53 wherein the source of ionizing radiation is a laser.

57. The apparatus of claim 53 wherein the apparatus is capable of analyzing nucleic acid materi-
10   als.

58. An apparatus for determining the nucleo-
tide sequence of a polynucleotide, comprising:
    (a) a mass spectrometer adapted to determine the relative molecular weights of individual single-
15   stranded nucleic acid molecules in a first plurality of different populations comprising a second plurality of single-stranded nucleic acid molecules, each molecule having a different molecular weight, and one defined terminus and one variable terminus, said variable
20   terminus terminating at a specific nucleotide, the variable terminus of each different first plurality of populations terminating at a different specific nucleo-
tide, wherein said molecules are generated from said polynucleotide; and
25   (b) a computer adapted to compare the molecu-
lar weights of each of said molecules in said different first plurality of populations to provide the nucleic acid sequence of said polynucleotide.

59. The apparatus of claim 58 further compri-
30   sing a laser adapted to generate light able to vaporize said populations of molecules.

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60. The apparatus of claim 58 further com-
prising a laser adapted to generate light able to ionize
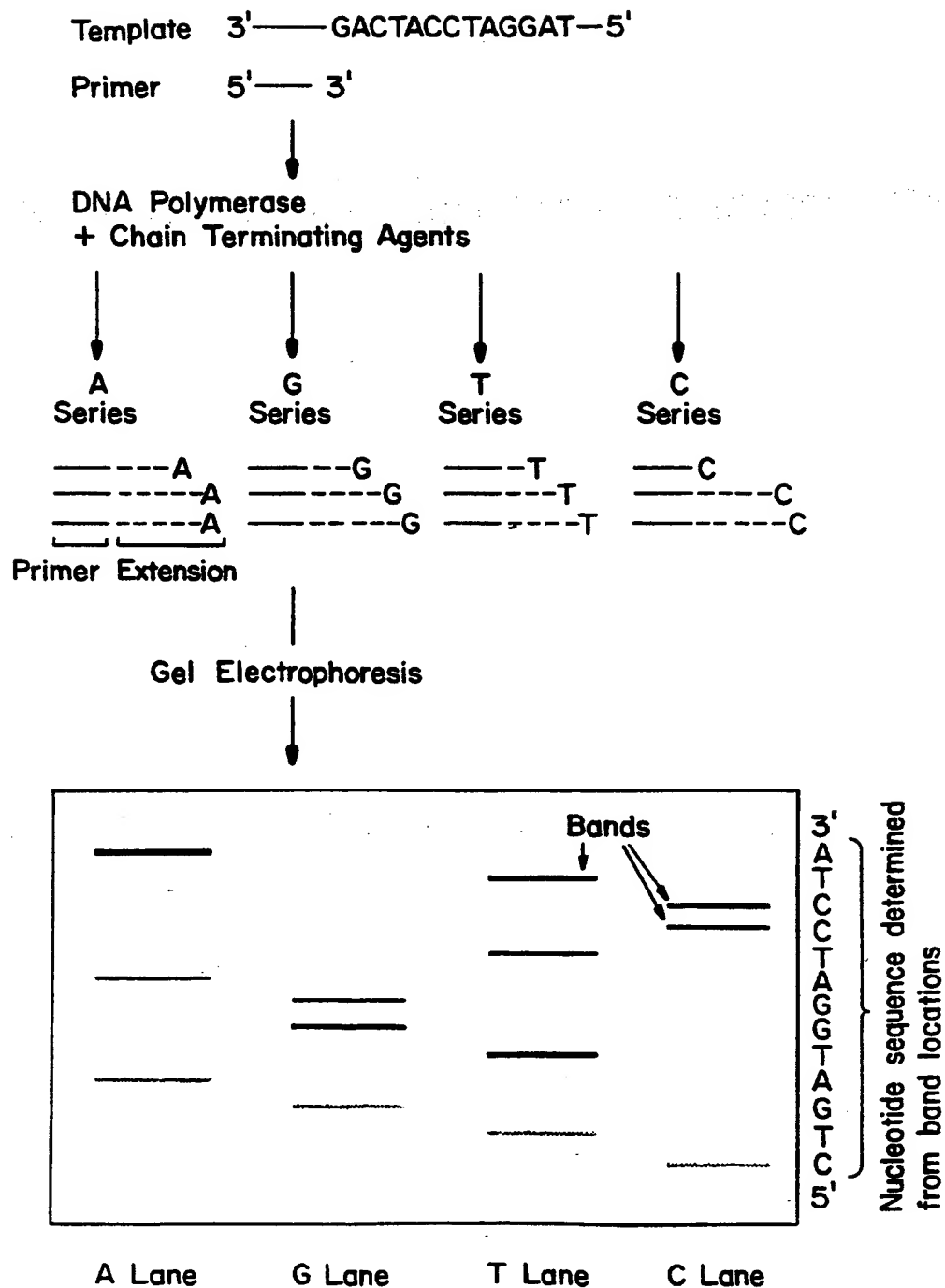said populations of molecules.

61. A population comprising a plurality of
5   single-stranded nucleic acid molecules, each molecule
having a different molecular weight and one defined
terminus and one variable terminus, said variable
terminus terminating at a specific nucleotide, said
molecules being in an ionic and vapor form.
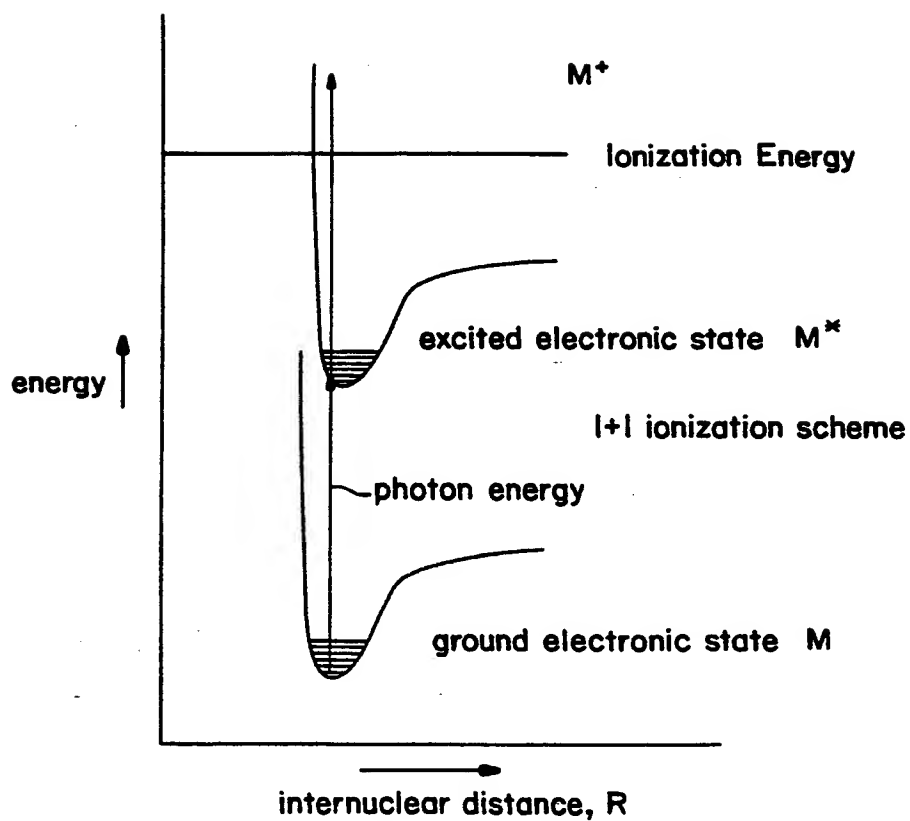
10   62. A plurality of populations of claim 61.

FIG. 1

Template   3'———GACTACCTAGGAT—5'

Primer   5'——— 3'

↓

DNA Polymerase
+ Chain Terminating Agents

↓   ↓   ↓   ↓

A   G   T   C
Series   Series   Series   Series

```
——— ---A      ——— --G       ——— -T        ——— C
————— ---A    ————— ---G     ————— ---T    ————— ---C
——— --- -A    ——————— ---G   ——— - ---T    ——————— ---C
```
└──┘ └────┘
Primer Extension

↓

Gel Electrophoresis

↓

Bands

```
3'
A
T
C
C
T
A
G
G
T
A
G
T
C
5'
```
Nucleotide sequence determined
from band locations

A Lane   G Lane   T Lane   C Lane

FIG. 2

FIG. 3

FIG. 4

30 cm focal length lens

14'

50'

20'

12'

FIG. 5

FIG. 6

quartz window

Iris

20 cm focal length telescope lense

S1 UV lenses
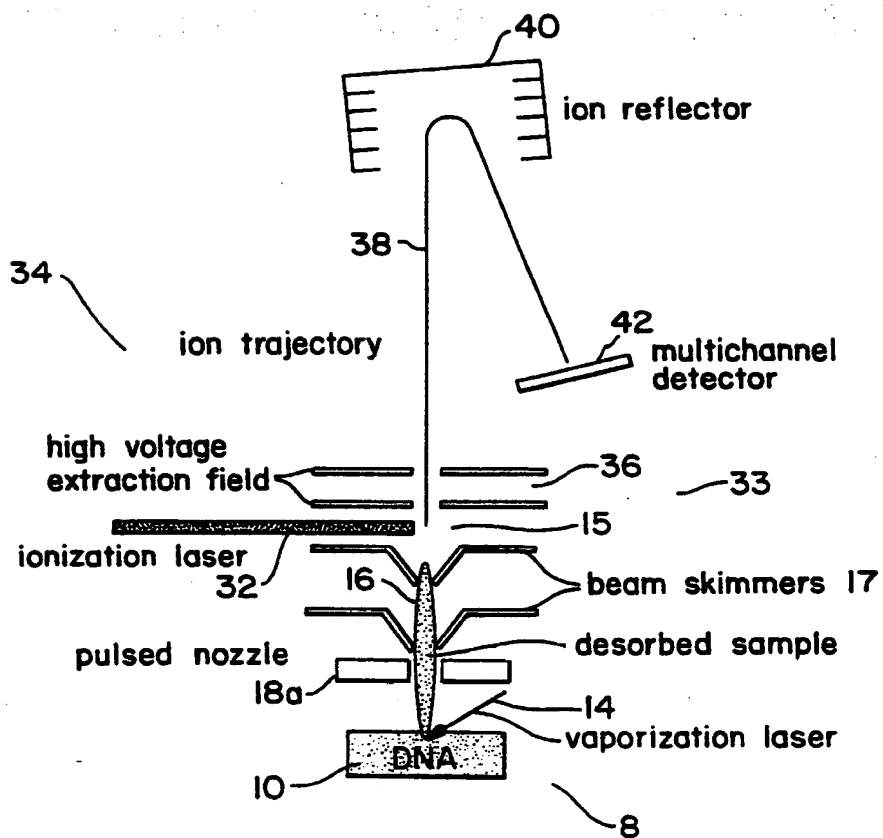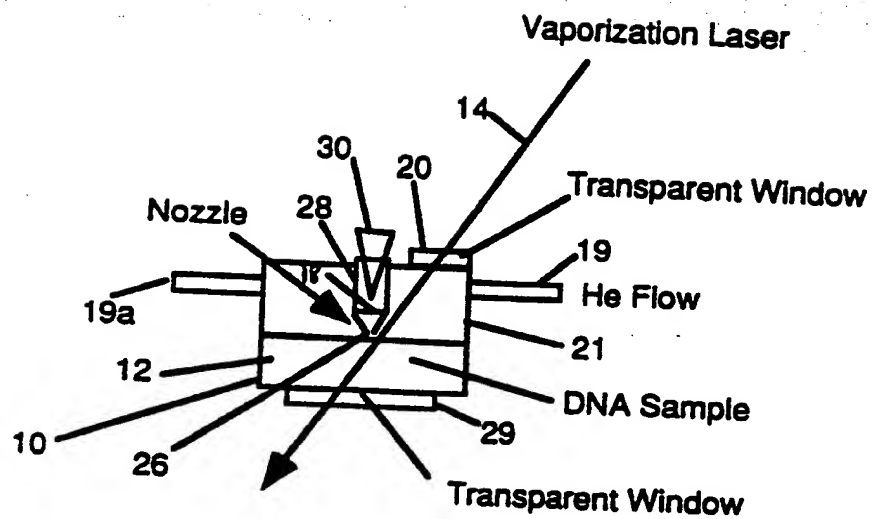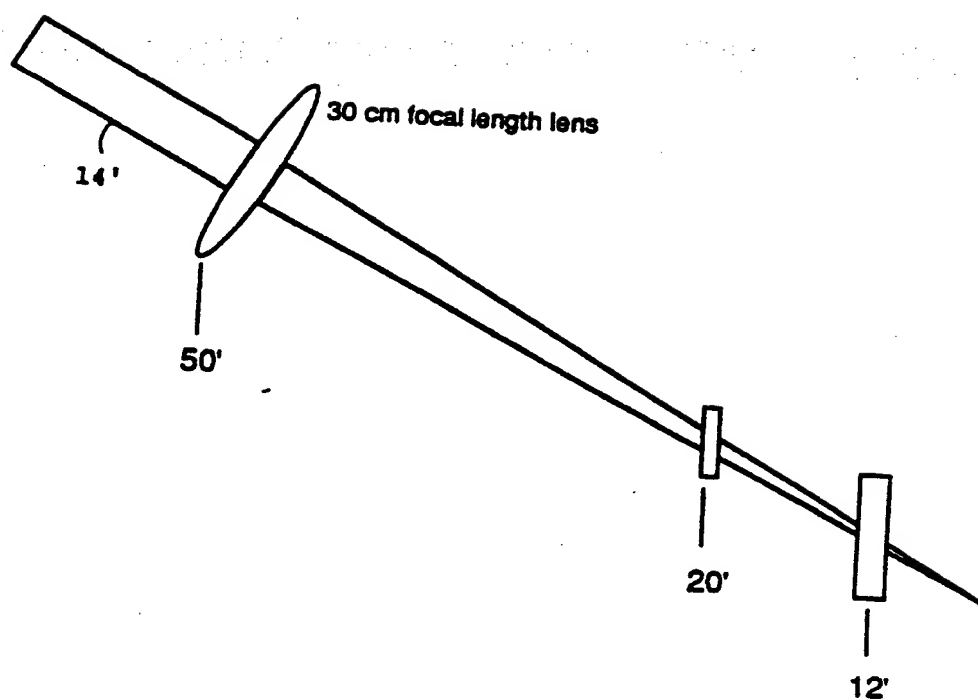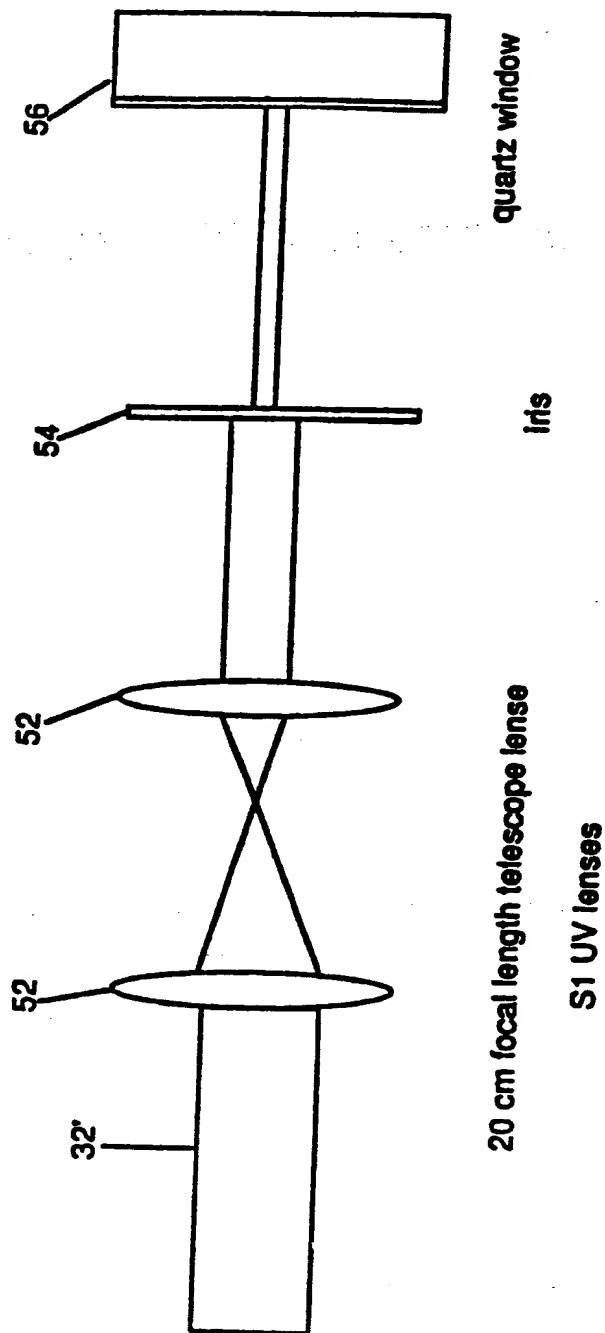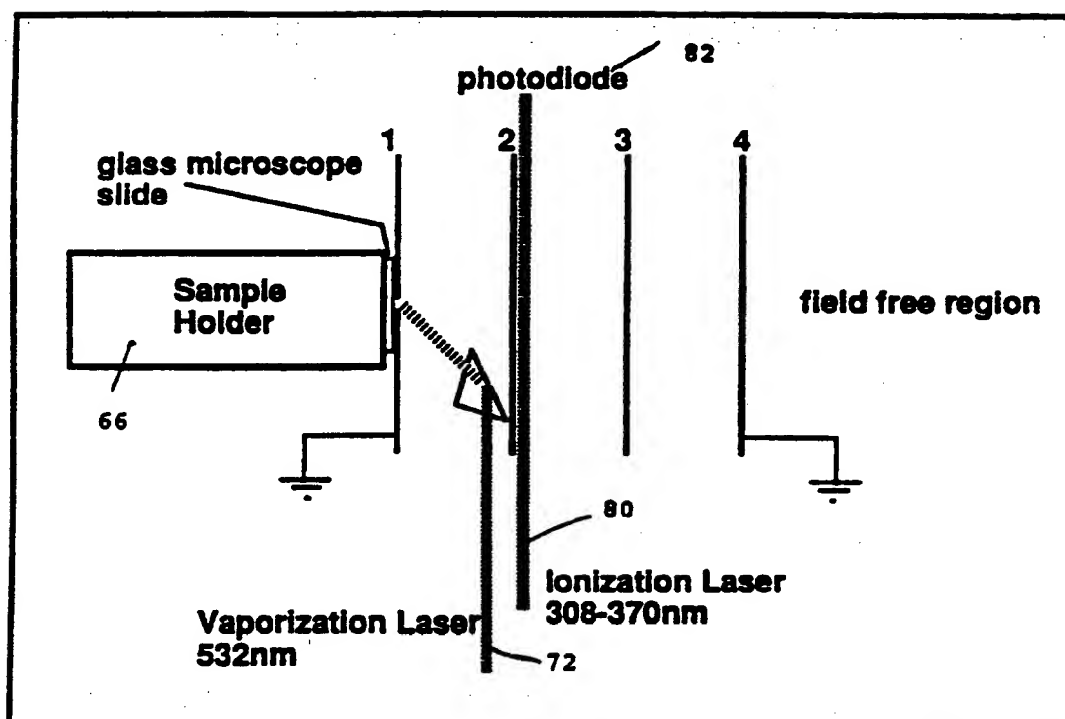
FIG. 7

LASER VAPORIZATION, TOF APPARATUS

Ion Detector

68

Ionization Guage

64

74

Single-Stranded
DNA Sample
In Rhodamine 6G
Thin Film

Sample
Holder

66

60

Turbo
Pump 62

63cm
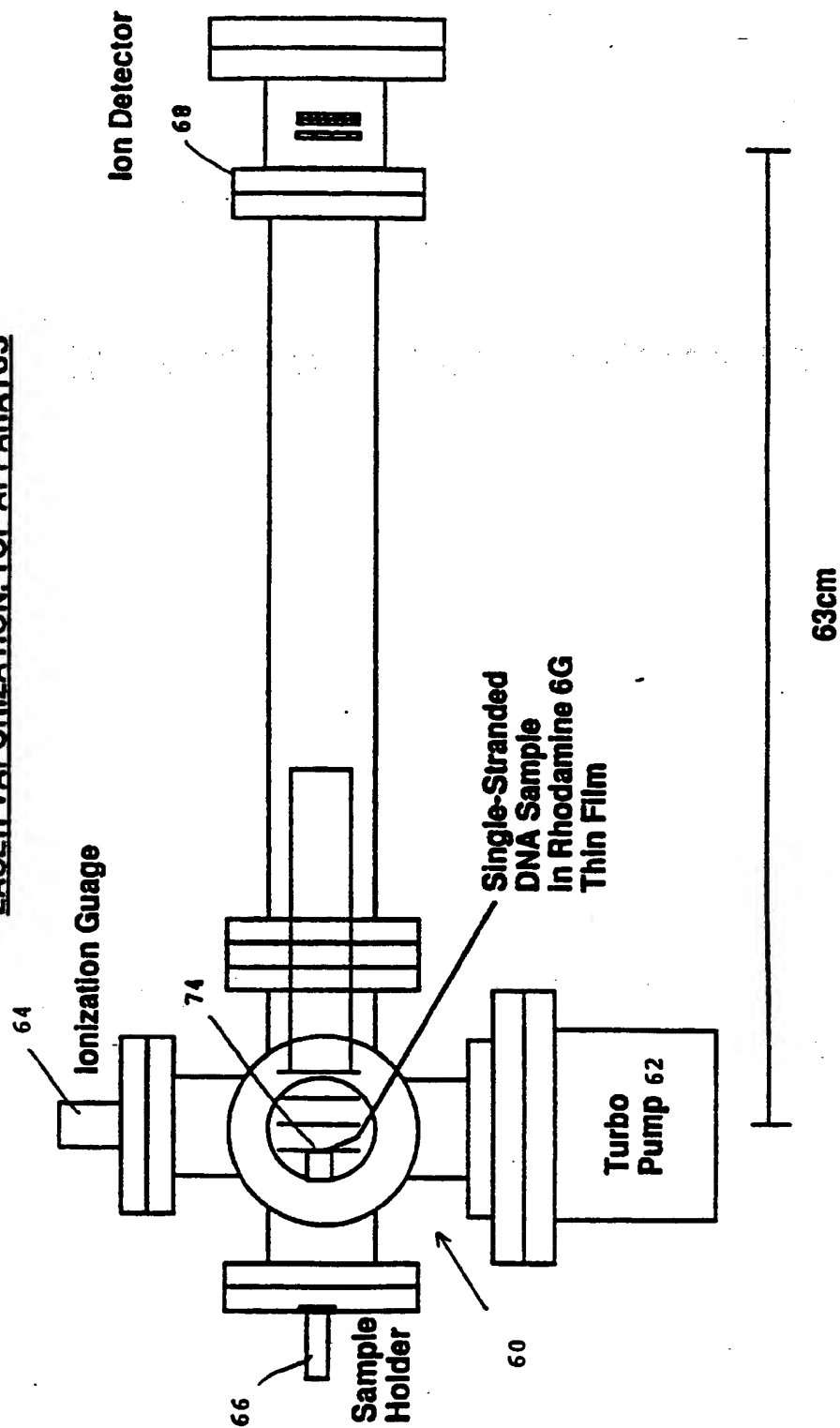
FIG. 8

FIG. 9

Time-of-Flight Mass Spectrum of Antracene-linked Thymidine-5'-monophosphate
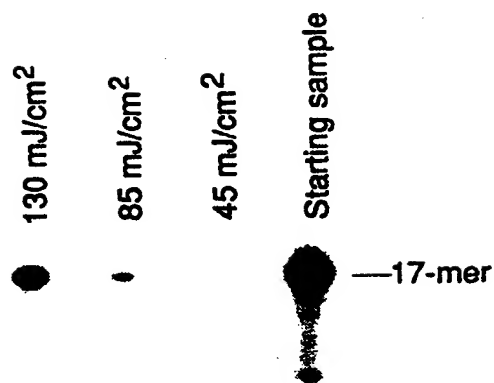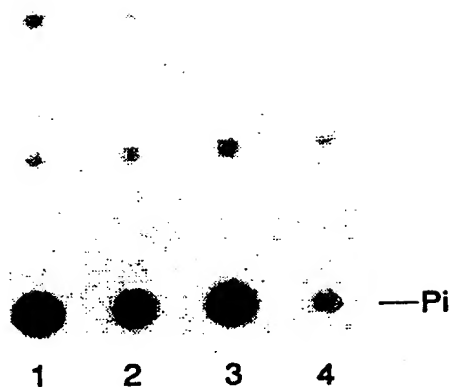
FIG. IOA

FIG. IOB

FIG. IOC

SUBSTITUTE SHEET

130 mJ/cm^2

85 mJ/cm^2

45 mJ/cm^2

Starting sample

—17-mer

FIG. 11

—1-mer

—Pi

1   2   3   4

FIG.12

^{32}P-Containing components recovered
(%).

FIG. 13A

FIG. 13 B

FIG. 14

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): B01D 59/44, H01J 49/00 U.S. CL. 250/282,287,288

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	250/282,287,288,423P

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A, 4,920,264 (BECKER) 24 April 1980, See Col 5, lines 13-47 and Fig 2.	1-5 45/1-46, 5-3-5-4.
X	US,A, 4,988,879 (ZARE ET AL) 29 January 1991, See Col. 7, lines 49-66.	6-9,26/1-29, 45/1-46, 48/1-49,53-57
X	Biomedical and Environment al Mass Spectrometry, Vol. 18, issued 1989, Karas et al., Ultraviolet – Laser Desurption/Ionization Mass Spectrometry of Femtomolar Amounts of Large Proteins, pp 841-843, See entire article.	10-12
A	US,A, 4,757,141 (FUNG ET AL) 12 July 1988, See entire documents.	
A	US,A, 4,855,225 (FUNG ET . AL) 08 August 1989, See entire document.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 May 1992	15 JUN 1992
International Searching Authority	Signature of Authorized Officer NGUYEN NGOC-XI INTERNATIONAL DIVISION Bruce C. Anderson
ISA/US	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers   , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)